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The Use of Molecular Descriptors To Model Pharmaceutical Uptake by a Fish Primary Gill Cell Culture Epithelium

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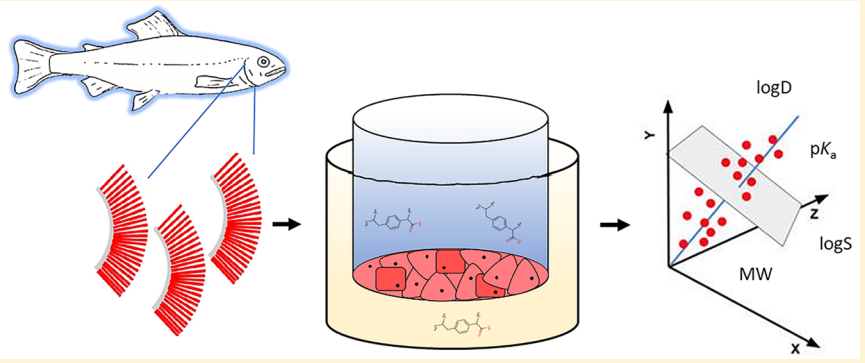
Supporting Information



ABSTRACT: Modeling approaches such as quantitative structure−activity relationships (QSARs) use molecular descriptors to predict the bioavailable properties of a compound in biota. However, these models have mainly been derived based on empirical data for lipophilic neutral compounds and may not predict the uptake of ionizable compounds. The majority of pharmaceuticals are ionizable, and freshwaters can have a range of pH values that affect speciation. In this study, we assessed the uptake of 10 pharmaceuticals (acetazolamide, beclomethasone, carbamazepine, diclofenac, gemfibrozil, ibuprofen, ketoprofen, norethindrone, propranolol, and warfarin) with differing modes of action and physicochemical properties (pK_a, log *S*, log *D*, log K_{ow}, molecular weight (MW), and polar surface area (PSA)) by an in vitro primary fish gill cell culture system (FIGCS) for 24 h in artificial freshwater. Principal component analysis (PCA) and partial least-squares (PLS) regression was used to determine the molecular descriptors that influence the uptake rates. Ionizable drugs were taken up by FIGCS; a strong positive correlation was observed between log *S* and the uptake rate, and a negative correlation was observed between pK_a, log *D*, and MW and the uptake rate. This approach shows that models can be derived on the basis of the physicochemical properties of pharmaceuticals and the use of an in vitro gill system to predict the uptake of other compounds. There is a need for a robust and validated model for gill uptake that could be used in a tiered risk assessment to prioritize compounds for experimental testing.

INTRODUCTION

Pharmaceuticals are biologically active molecules that have been detected in surface waters at nanogram to microgram per liter concentrations[1] and are widely reported in aquatic fauna.[2] The impacts associated with pharmaceutical exposure of aquatic organisms is unclear, and knowledge is necessary to inform regulatory authorities and the pharmaceutical industry of compounds that may pose a risk.[3]

As part of a chemical risk assessment, it is necessary to determine the likelihood of bioaccumulation. A bioconcentration factor (BCF) is a measure which includes uptake ($k1$) and elimination rates ($k2$) and internal steady state concentration.[4,5] However, the uptake process, along with metabolism, represents the largest factors of uncertainty in fish bioaccumulation models,[6,7] and BCF values for individual compounds derived from in vivo studies can vary substantially.[8] Consequently, a novel approach to evaluate the bioavailability properties of a chemical has been proposed which utilizes nonguideline methodologies in a tiered risk assessment.[8] In this approach, in silico or in vitro data may be used in the lower tiers to assess a chemical's bioavailability; if

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Table 1. Pharmaceutical Molecular Descriptors

	pK_a	log S	molecular wt (g mol^{-1})	log D	log K_{ow}	PSA (Å^2)	% ionization at pH 7.6
acetazolamide	7.20	−2.36	222.24	0.23	3.48	115.04	71.5
beclomethasone	13.85	−5.4	408.92	4.16	3.49	106.97	0
carbamazepine	15.96	−3.2	236.27	2.28	2.28	46.33	0
diclofenac	4.00	−4.8	296.15	1.22	1.9	52.16	100
gemfibrozil	4.42	−4	250.33	1.40	4.77	46.53	99.9
ibuprofen	4.91	−3.5	206.28	0.29	2.48	40.10	99.8
ketoprofen	4.45	−4.1	254.28	0.06	0.97	54.37	99.9
norethindrone	17.59	−4.7	298.40	2.98	3.15	37.30	0
propranolol	9.42	−3.5	259.34	1.29	3.09	41.49	98.5
warfarin	5.08	−3.8	308.32	0.16	0.85	63.60	99.7

there is enough information to classify bioaccumulation potential, then a decision can be made as to whether further BCF studies are required.[8]

Many mechanistic assessments of contaminant uptake are based on in silico models, such as quantitative structure−activity relationships (QSARs), that have largely been derived from data for lipophilic neutral compounds that passively diffuse across lipid membranes and undergo little to no metabolism.[6] QSARs can include linear-based estimations or more recently, machine learning applications such as neural networks and tree-based learning to predict organic chemical bioconcentration.[9] However, an estimated 77.5% of pharmaceuticals are ionizable.[10] Thus, the applicability of the QSAR models developed on other contaminant classes (i.e., neutral hydrophobic contaminants) may be limited leading to inaccurate estimation of the accumulation of pharmaceuticals. For compounds that are ionizable, the acid−base dissociation constant (pK_a) describes the dissociation of the drug at a given pH and influences solubility, lipophilicity, permeability, and protein binding.[11] In the aquatic environment, surface water pH will determine chemical speciation, and this is predicted to have an influence on bioavailability.[12] The typical pH values of environmental water range between 6 and 9,[12] although fish can be found in bodies of water that are extremely acidic (pH 3)[13] and highly alkaline (pH 10.5).[14] The effects of pH on the toxicity[12] and uptake/elimination of ionizable compounds in fish have been demonstrated.[15−17] Recently, Bittner et al.[18] demonstrated the impact of pH (5.5−8.6) on the uptake and toxicity of beta-blocker pharmaceuticals in zebrafish larvae; where the skin is likely to be the significant route of uptake. Karlsson et al.[19] examined the effects of water and sediment pH (5.5−8.5 pH) on the uptake of three pharmaceuticals with a range of pK_a (4.01−9.62 pK_a) in the freshwater oligochaete *Lumbriculus variegatus*.

There is a desire to develop in vitro models that replace or supplement current animal experimental procedures[20] in accordance with the replacement, reduction, and refinement (3Rs) principle.[21] This is also reflected in European legislature which states that non-animal alternative approaches should be used in place of animal procedures wherever possible. A fish gill cell culture system (FIGCS) was developed using primary fish cells which has shown promise as an alternative system for whole fish chemical uptake studies.[22] FIGCS maintains many of the characteristics of the in vivo epithelium, including the presence of multiple cell types associated with transport of ions across the gills and the ability to tolerate freshwater−water application to the apical surface. The in vitro data obtained from FIGCS experiments has the potential to be an important component of the lower tier in a tiered testing system[8] as the gills are a primary route of uptake in fish.[22]It has recently been used to investigate the absorption of seven pharmaceuticals with a similar pK_a of 8.1 to 9.6 across the gill.[23]

There is a paucity in fish pharmaceutical uptake and BCF values because the tests to derive these use a large number of organisms, are time-consuming, and are expensive to conduct. In this study, we assessed the uptake of 10 pharmaceuticals by an in vitro fish gill model with differing physiochemical properties and there were three aims. The first aim was to assess the uptake of ionizable pharmaceuticals with this fish gill epithelium. The second aim was to demonstrate how an in vitro epithelial model can be used to evaluate the propensity of a drug to enter a fish from the water and how this information could become part of a tiered risk assessment approach.[8,24] Lipsinki et al.[25] proposed that the molecular properties (molecular weight, hydrogen bond donors and acceptors, and log K_{ow}) of a chemical can be used as a screening tool to determine the likelihood of absorption across a membrane; our third aim was to extend this concept and use the pharmaceutical molecular descriptors and partial least-squares (PLS) regression analysis to model uptake rate and identify those descriptors which influence gill uptake. We were able to show that solubility, pK_a, octanol−water distribution coefficient at pH 7.4, and molecular weight are the most important descriptors that drive epithelial drug uptake rates.

MATERIALS AND METHODS

Fish Gill Cell Culture System (FIGCS). Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from a local trout farm and housed in dechlorinated−aerated, London city tap water ($[Na^+]$ = 0.53 mM, $[Ca^{2+}]$ = 0.92 mM, $[Mg^{2+}]$ = 0.14 mM, $[K^+]$ = 0.066 mM, and $[NH_4^+]$ = 0.027 mM). The temperature was maintained at 14 °C with a 14 h light:10 h dark cycle, and fish were fed a 1% (w/v) ration of trout pellets daily.

Primary fish gill cell culture inserts were prepared in companion wells and maintained according to protocols described in Schnell et al.[22] The transepithelial resistance (TER) was monitored daily using an epithelial tissue voltohmmeter (EVOMX) with STX-2 chopsticks (World Precision Instruments). A TER value of above 3000 Ω cm^{-2} was used as criteria for the presence of a tight epithelium, as previously determined using ^{14}C-mannitol as a paracellular permeability marker.[23]

Pharmaceuticals' Exposures and Cell Viability Assay. Analytical grade pharmaceuticals (purity ≥97%) from differing classes of action with differing chemical properties (Table 1) were purchased from Sigma-Aldrich and included acetazolamide (CAS: 59-66-5), beclomethasone (CAS: 4419-39-0),

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carbamazepine (CAS: 298-46-4), diclofenac sodium salt (CAS: 15307-79-6), gemfibrozil (CAS: 25812-30-0), ibuprofen sodium salt (CAS: 31121-93-4), ketoprofen (CAS: 22071-15-4), norethindrone-19 (CAS: 68-22-4), propranolol hydrochloride (CAS: 318-98-9), and warfarin (CAS: 81-81-2). Pharmaceutical stocks were prepared at a concentration of 1 mg mL^{-1} in methanol or ethanol and stored at −80 °C.

Following the formation of a tight epithelium, inserts were prepared for exposure by washing with phosphate buffered saline. The apical freshwater (AFW) used for apical exposure was prepared according to the $OECD_{203}$ test guidelines[26] (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.8 mM $NaHCO_3$, 77.1 μM KCl, with a measured pH 7.6) with individual pharmaceuticals added at a concentration of 1 μg mL^{-1}, which is equivalent to 450 nM acetazolamide, 245 nM beclomethasone, 423 nM carbamazepine, 338 nM diclofenac, 399 nM gemfibrozil, 485 nM ibuprofen, 393 nM ketoprofen, 355 nM norethindrone, 386 nM propranolol, and 324 nM warfarin. To expose the cells, 1.5 mL of exposure water was added to the apical compartment and 2 mL of L15 media with 5% FBS was added to the basal compartment. The inserts and the 1.5 mL exposure water samples (T_0) were incubated at 18 °C in the dark for 24 h. In the case of the T_0 samples, this was to assess if the compounds remained stable over the 24 h exposure period at 18 °C in the absence of cells. The T_0 and the 1.5 mL apical compartment water samples after a 24 h exposure (T_{24}) were collected and stored at −80 °C for further analysis. Measurements were made on four inserts derived from two to three biological replicates, with each biological replicate comprising of cells harvested from two fish.

To consider the adhesion of the compounds to the companion well and insert membrane during the 24 h of exposure, a cell-free experiment was performed. To assess pharmaceutical toxicity, single seeded primary gill cells were grown in T75 flasks to 80% confluence and then trypsinized and transferred to the 96-well plates at a density of 1×10^5 cells $well^{-1}$. Twenty-four hours postseeding in the 96-well plates, cells were exposed to pharmaceuticals at 1 μg mL^{-1} in L15 with 5% FBS for 24 h, after which a MTT viability assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed following methods adapted from Riss et al.[27] The pH stability of the AFW with 1 μg mL^{-1} of compound was measured with and without cells and found to be stable over a 24 h period.

HPLC Analysis. For HPLC analysis, individual T_0 or T_{24} samples were pooled into three separate mixtures: mix A included beclomethasone, ibuprofen, and warfarin, mix B included carbamazepine, diclofenac, gemfibrozil, ketoprofen, and norethindrone, and mix C included acetazolamide and propranolol for solid phase extraction (SPE) with Oasis HLB cartridges (200 mg sorbent, 6 cm^3). Cartridges were initially conditioned with 6 mL of methanol (HPLC grade) followed by 6 mL of water (HPLC grade) and then loaded with pooled sample mix A, B, or C. Cartridges were washed with 4 mL of water, dried under vacuum pressure, and eluted with 6 ml of methanol or stored at −80 °C for later elution. The samples were then dried under nitrogen at 45 °C for 80 min (Biotage TurboVap), reconstituted in 500 μL of 90:10 (v/v) water/acetonitrile, and vortexed for 2 min before transfer to amber HPLC vials for analysis.

Liquid chromatography was performed on an Agilent 1260 Infinity series LC system using a Waters Sunfire C_{18} column (100 Å, 3.5 μm, 2.1 mm × 150 mm) at a flow rate of 0.2 mL min^{-1} and an injection volume of 20 μL. Mobile phases A and B consisted of HPLC grade water and HPLC grade acetonitrile, respectively, with initial running conditions of 10% phase B at a column temperature of 40 °C. The gradient elution was as follows: linear ramp with phase B increased to 80% at 12 min, held for 13 min, and then returned to initial conditions at 28 min. The total run time was 40 min including a 12 min re-equilibration period. An Agilent 1290 Infinity Diode Array Detector was used for the detection of diclofenac and warfarin at 214 nm; carbamazepine, ibuprofen, and gemfibrozil at 220 nm; beclomethasone and propranolol at 230 nm; norethindrone at 254 nm; ketoprofen at 263 nm; and acetazolamide at 273 nm.

Method performance was assessed by matrix-matched calibration curves generated for the AFW. Method linearity (5 concentrations, $n = 3$) was determined from 0.5 to 2.5 μg mL^{-1}, and signal-to-noise ratios of 3:1 and 10:1 of low concentration spiked samples were used to determine the LOD and LOQ, respectively ($n = 6$). Precision was determined using spiked samples at 1 μg mL^{-1} ($n = 6$), and accuracy was determined using spiked samples and values from method linearity ($n = 6$). Recovery was assessed by comparing spiked samples (pre-extraction) to postextract spiked samples at a concentration of 0.5, 1, or 2 μg mL^{-1} ($n = 3$).

Estimation of Gill Uptake Rates. Primary gill cell culture pharmaceutical uptake was calculated based on the loss of compound from the apical compartment corrected for the amount that adhered to the polystyrene plastic of the companion wells and inserts without cells over 24 h (eq 1)

$$\text{Uptake rate (nmol cm}^{-2}\text{ h}^{-1}) = (T_0 - T_{24}) - (T_0^{\,P} - T_{24}^{\,P})]/(t \times \text{cm}^2) \quad (1)$$

where T_0 and T_{24} represent the moles (nmol) of drug present in the apical compartment in the presence of cells at 0 and 24 h, respectively, and $T_0^{\,P}$ and $T_{24}^{\,P}$ represent the moles of drug present in the apical compartment in the absence of cells at 0 and 24 h, respectively; t = time of the flux measurement (24 h), and cm^2 represents the surface area of the epithelium (0.9 cm^2). Sorption controls (inserts and exposure media only) were setup to account for any losses of compound through volatilization, sorption to plastics, and any other degradative processes. Insert controls showed that these processes were negligible, and therefore, in the presence of cells, the disappearance of a compound is related to the uptake of the compound into the gill epithelium and its transfer across into the basolateral layer over 24 h.

Statistics and Modeling Approaches. A one-way ANOVA followed by a Tukey's post hoc test was performed to compare the uptake rates of each compound using GraphPad Prism 6.0. Modeling approaches used six molecular descriptors (Table 1) including the acid dissociation constant (pK_a), the octanol−water distribution coefficient at pH 7.4 (log D), the octanol−water partition coefficient (log K_{ow}), polar surface area (PSA), and molecular mass (M_w). The two descriptors log K_{ow} and log D are both measures of hydrophobicity, but log D takes into account both neutral and ionizable species at a given pH whereas log K_{ow} only takes into account the neutral fraction. Principal component analysis (PCA) and partial least-squares (PLS) regression were performed using the R statistical computing language, R version 3.4.3 (freely available at https://www.r-project.org/). All scripts were written with RStudio (freely available at

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https://www.rstudio.com/); packages used for PCA and PLS analyses were *stats* and *plsdepot*, respectively. The full data set used in modeling, latent variable scores, loadings, weights, and cross-validations of models are given in Figure S3 and Tables S4–S7). For cross-validation of the PLS model, a leave-one-out approach was used.

RESULTS

Cell viability was assessed by MTT assay and none of the pharmaceuticals at a concentration of 1 μg mL^{-1} showed signs of cytotoxicity (Figure S1); HPLC method performance assessment is provided in Tables S1 and S2.

Pharmaceutical adhesion to the companion wells over 24 h was between 0.7 and 5% (data not shown) and was taken into consideration when uptake rates were calculated. Acetazolamide uptake (0.125 ± 0.032 nmol cm^{-2} h^{-1}) was significantly greater than those of beclomethasone, carbamazepine, diclofenac, and norethindrone (beclomethasone, 0.021 ± 0.015 nmol cm^{-2} h^{-1}; carbamazepine, 0.022 ± 0.004 nmol cm^{-2} h^{-1}; norethindrone, 0.024 ± 0.003 nmol cm^{-2} h^{-1}; diclofenac, 0.027 ± 0.003 nmol cm^{-2} h^{-1}) (Figure 1). The

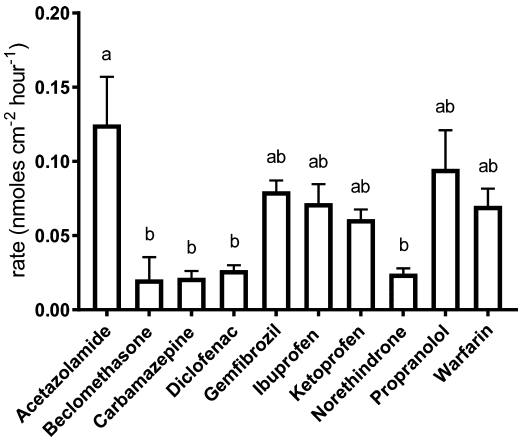

Figure 1. Pharmaceutical uptake rate into the fish gill cell culture system. Values represent average of four inserts derived from 2 to 3 biological replicate, with each biological replicate comprising of cells from 2 fish. Bars with differing letters are significantly different from each other when compared via a one-way ANOVA followed by a Tukey's posthoc test; $p < 0.05$.

other ionizable drugs, except for diclofenac, showed higher, but not significantly higher, uptake rates (ibuprofen, 0.072 ± 0.013 nmol cm^{-2} h^{-1}; gemfibrozil, 0.075 ± 0.007 nmol cm^{-2} h^{-1}; ketoprofen, 0.061 ± 0.006 nmol cm^{-2} h^{-1}; propranolol, 0.095 ± 0.026 nmol cm^{-2} h^{-1}; and warfarin, 0.070 ± 0.012 nmol cm^{-2} h^{-1}) compared to the neutral drugs beclomethasone, carbamazepine, and norethindrone (Figure 1 and Figure S2).

Modeling of the molecular descriptors was performed using PCA analysis to identify compound similarity (Figure 2). The first two principal components explained a cumulative variance of 69% (PC1 = 48%, PC2 = 21%) in the descriptor space. The score plot indicates that there were no apparent outliers in the data set. Clustering of compounds was minimal but was expected with the low number of cases available for modeling (n = 10). The largest variation in the descriptor space was observed for the compound beclomethasone (Figure 2). The variance of this case can be explained in terms of the loadings, where this compound was the largest (MW = 408.92) and most hydrophobic (log D = 4.16) of all the compounds that

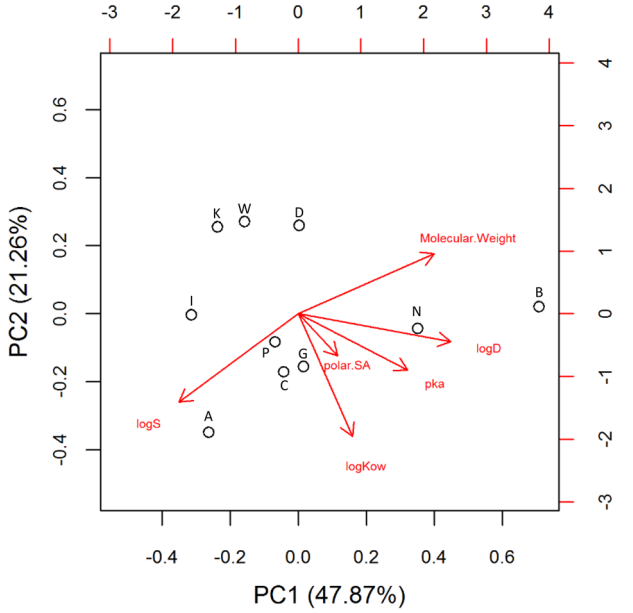

Figure 2. PCA biplot showing the first two principal component (PC1 and PC2) loadings and scores for each molecular descriptor and case, respectively. Scores are indicated on the first axes (left and bottom, black); loadings are indicated by the second axes (right and top, red). A, acetazolamide; B, beclomethasone; C, carbamazepine; D, diclofenac; G, gemfibrozil; I, ibuprofen; K, ketoprofen; N, norethindrone; P, propranolol; W, warfarin.

were tested. From the descriptor loadings, log S and MW were negatively correlated with each other. The loadings for the first latent variable also showed that log D (0.567), MW (0.505), log S (−0.444), and pK_a (0.407) were more important variables than log K_{ow} (0.201) or PSA (0.146).

PLS was implemented to interpret molecular descriptors that influence gill uptake rates of pharmaceuticals and enable a predictive modeling approach with

$$\begin{aligned}\text{Uptake rate (nmol cm}^{-2}\text{ h}^{-1}) &= 1.23\text{E-}01 + (-1.59\text{E-}03 \times \text{p}K_a) + (1.53\text{E-}02 \times \log S) \\ &+ (-7.26\text{E-}03 \times \log D) + (9.15\text{E-}03 \times \log K_{ow}) \\ &+ (-9.48\text{E-}05 \times \text{MW}) + (3.83\text{E-}04 \times \text{PSA}) \qquad (2)\end{aligned}$$

The adjusted correlation coefficient (R^2_{adj}) and the cross-validated R^2 (Q^2) of the PLS regression model were 0.7863 and 0.5397, respectively. No cases were observed as outliers in the PLS model determined by the Hotelling's T^2 95% confidence ellipse (data not shown). On the basis of the cumulative Q^2 statistic (see Figure S4), the optimal number of latent variables for the PLS model was two. The loadings plot (Figure 3a) indicated that log S was positively correlated with gill uptake whereas log D, pK_a, and MW were negatively correlated with gill uptake. The log K_{ow} and PSA descriptors were relatively less important in modeling gill uptake when compared with the previously mentioned descriptors. The use of PLS to predict gill uptake showed good performance with the mean absolute error of 0.01 ± 0.01 nmol cm^{-2} h^{-1} (MAE ± SD) for all the compounds tested. Larger inaccuracies in the predictions were observed for the four compounds: carbamazepine (122%), diclofenac (61%), norethindrone (35%), and propranolol (32%) (Figure 3b,c).

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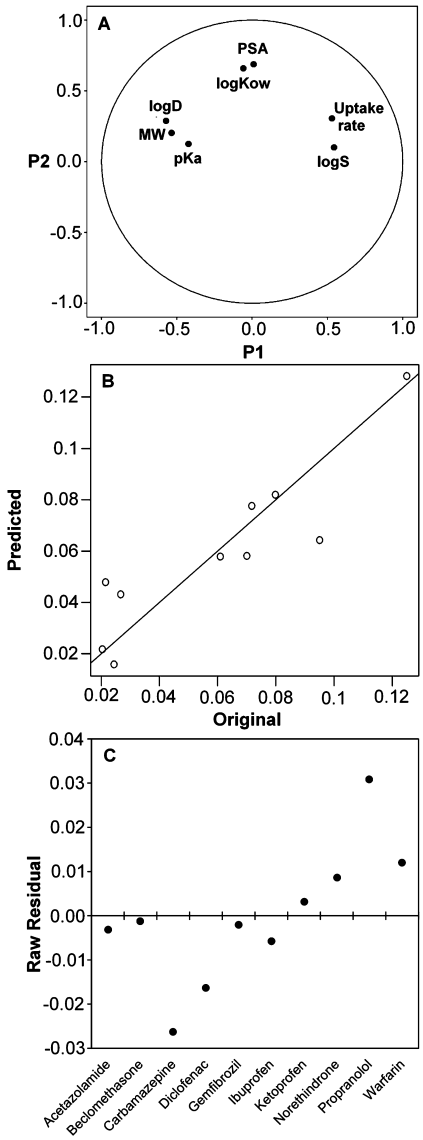

Figure 3. PLS regression analysis showing (A) loadings of the first two latent variables for molecular descriptors (independent variables) and uptake rate (dependent variable), (B) predicted versus observed gill uptake rates using PLS regression model, and (C) raw residuals of predicted uptake rates.

DISCUSSION

The uptake rate of 10 pharmaceuticals by a fish primary gill cell culture system was assessed. From our data set, we were able to demonstrate that a PLS regression model based on the drug molecular descriptors could be developed for pharmaceutical uptake rate by this epithelium, with log *S*, pK_a, log *D*, and MW found to be the most important descriptors that drive epithelial drug uptake rates.

The uptake rates of the compounds from the apical compartment reflect the apparent epithelial permeability (P_{app}) of the compound by the FIGCS cells. The P_{app} can be expressed as an equation (eq 3) and takes into consideration four factors: partitioning in the aqueous boundary layer (P_{ABL}, ABL), adhesion to filter insert (P_f), and transcellular (trans) or paracellular (para) transfer (P_{trans}, P_{para}).[28] The aqueous boundary layer is assumed to have a distinct boundary with the bulk water adjacent to both sides of the membrane.[29]

$$\frac{1}{P_{app}} = \frac{1}{P_{ABL}} + \frac{1}{P_f} + \frac{1}{P_{trans} + P_{para}} \quad (3)$$

Two of the four factors can be discounted due to the nature of the system. First, adhesion to plastic (P_f) and inserts was taken into consideration when calculating the uptake rate. Second, a previous study conducted using the paracellular marker ^{14}C-mannitol determined that at TER values above 3000 Ω cm^2, the FIGCS were relatively impermeable to the marker and indicated that transport was via transcellular routes;[23] all compounds in the current study had a greater molecular weight than mannitol and thus paracellular transfer (P_{para}) was negligible. Thus, the uptake rates reflect partitioning in the ABL (P_{ABL})and uptake into cells and across the basolateral membrane (P_{trans}) into the basolateral compartment in addition to any potentially metabolized compound efflux from the gill cells back into apical compartment.

All uptake studies were conducted in AFW, and under these conditions acetazolamide (71.5%), diclofenac (100.0%), gemfibrozil (99.9%), ibuprofen (99.8%), ketoprofen (99.9%), propranolol (98.5%), and warfarin (99.7%) are all predicted to be ionized (% ionization is indicated in parentheses), whereas beclomethasone, carbamazepine, and norethindrone are not ionized. All ionizable drugs, except diclofenac, showed higher permeation into the primary gill cell epithelium when compared to the neutral drugs (Figure 1). The observation that ionizable drugs are capable of permeating the gill epithelium corroborates a previous study in FIGCS concerning the uptake of a set of pharmaceuticals with pK_a between 8.1 and 9.6,[23] and a number of studies suggest that ionizable compounds can be taken up by the fish gill.[15,16,18,19,23,30,31] The uptake of nine weakly acidic chlorinated phenols by rainbow trout did not vary between pH 6.3 and 8.4 despite the proportion of the compounds that ranged in ionization from 1 to 99%;[15] the accumulation of the weak basic diphenhydramine (pK_a 9.1) at 10 μg L^{-1} reached a steady state in fathead minnow at pH 7.73 and 8.63 after ∼24 h, and only at pH 6.87, was accumulation greatly reduced[31] and ionizable surfactant,[32] perfluoroakyl acids,[33] and phenols and carboxylic acids[34] were observed to cross the gills of fish. However, membrane permeation may be an order of magnitude less than that for the neutral form.[30] Erickson et al.[16] developed a mechanistic model of ionized organic chemical uptake at the fish gill which expanded on an original model for un-ionized chemical uptake.[35,36] This new model included a factor that takes into account the ability of the fish to alter the pH adjacent to the apical membrane thus generating a microclimate that differs from the bulk water.[37] These changes in pH at the gill surface helped to explain the uptake of diphenylamine[31] and the chlorinated phenols.[15,16] However, in the current study, uptake of the acidic and basic pharmaceuticals showed similar uptake rates, and if uptake is solely due to the neutral form of the drug, then the pH of the culture epithelial microclimate would have to be in the region of pH 3 to ensure that the weakly acidic drugs (pK_a 4−5.08) were un-ionized. It is also unlikely that uptake is solely due to the ionized form because the basic drugs show similar uptake rates (Figure 1 and reference 23). In contrast to the other ionizable compounds, diclofenac is the only drug that exhibited a relatively lower uptake rate. It is unclear why this may be; but of the drugs used in the current study, the structure of diclofenac is more complex, containing both an amine and carboxylic acid group, and lacks

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conformational flexibility[38] which may influence transport by the gill epithelium.

The PLS modeling approach showed that all descriptors here have an influence on the uptake rate, but more of the explained variance was correlated to the log *S*, log *D*, pK_a, and MW descriptors. The regression model (eq 2) showed a good potential to predict uptake rates in the FIGCS system at the tested concentration and water chemistry (r^2 = 0.786). Modeling is an important aspect in understanding the fate of pharmaceuticals in the aquatic environment, and these approaches are complementary to in vitro systems for the replacement of animal testing. Comparison to in vivo fish uptake rates would be useful; however, there are a limited number of studies reporting these values. We have collated the '*steady state*' plasma concentrations for 9 of the 10 pharmaceuticals in Table S8; but note that the complexity and variation in pH, exposure, species, size, and temperature make a direct comparison to our data difficult. Furthermore, these studies do not allow us to derive uptake rates and therefore are not suitable for comparison with our data set. Predicted K_1, LC_{50}, and BCF values can be derived from QSAR models for fish (Table S8). However, a poor correlation was observed between the predicted K_1, LC_{50}, and BCF values and our in vitro pharmaceutical uptake rates (Table S8), emphasizing the need for alternative models for these compounds. To fully validate the model, a much larger number of compounds would be needed. A robust and validated model for gill uptake could then be used as a prescreen to prioritize compounds for experimental testing in a tiered approach.[8] In this scenario, if a compound is predicted to not be bioavailable in in vitro studies and other information from lower tier screens support this observation, then further BCF testing in living fish may not be required.[8,24]

The pharmaceutical uptake rate was most strongly positively correlated to log *S* (Figure 2b) suggesting that this physiochemical property facilitates access of the pharmaceuticals to the cells and uptake. The ABL in multiwell plates is between 1000 and 2000 μm and forms a significant diffusional barrier[39] whereby the concentration in bulk solution exceeds that located at the membrane surface. Increased solubility aids permeability across the ABL[29] allowing for greater interaction with the membrane.[40] Carbamazepine was expected to have a higher uptake based on solubility as well as being neutral and hydrophobic, but influx rates were low, and carbamazepine had the largest prediction inaccuracy in the PLS regression model. The reason for this is uncertain. Carbamazepine has a low BCF value in adult zebrafish (BCF_{ss} of 1.41 ± 7.13 L kg^{-1}), but this is likely associated with greater biotransformation capacity and clearance rather than a significant reduction in uptake when compared to other pharmaceutical and personal care products tested.[17] Whether the gills actively excrete carbamazepine back into the apical water compartment remains to be determined. Carbamazepine's mode of action is promiscuous, and it interacts with different types of receptors and channels.[41] However, the main targets are voltage-gated Na^+ channels located on the surface of the cells[41] where carbamazepine acts as a competitive inhibitor by allosteric inhibition.[42] A possibility is that in our system, the drug adheres to and interacts with the surface and related channels but does not permeate into the cell.

A negative correlation of uptake rates with log *D* was observed. The gill membrane consists of a range of phospholipids (e.g., phosphatidylethanoilamines and phosphatidylcholine) with differing properties capable of forming electrostatic and hydrogen bonds with charged molecules. It has been shown that ionized drugs can partition into artificial lipid membranes greater than predictions based on log *K*ow,[44] and there is a positive relationship between the dipole potential in the region between the aqueous phase and the interior membrane bilayer allowing permeation of ionized compounds in these synthetic membranes.[24,45,46] This phenomenon gave rise to the pH-piston hypothesis to explain sorption of ionized drugs into artificial vesicles consisting of dioleylphosphatidylcholine, due to electrostatic interactions with acidic and basic drugs,[45] and may explain how the ionized compounds are able to cross the membrane.

The PLS regression also indicated that the uptake rates were negatively correlated with MW. MW is known to play a distinct role in cellular uptake of solutes and has been used successfully to model permeability of both neutral and charged molecules; this is in addition to being a component of Lipinski's rule of five in drug discovery.[43,47,48] The log K_{ow} and PSA accounted for some of the variance in the regression but to a much lesser extent than the other molecular descriptors.

The role of transport proteins in ionizable drug uptake is axiomatic,[49−51] but the extent of the role that transport proteins play in drug uptake is debated. Kell and colleagues proposed that uptake is almost solely due to transport proteins,[52] though this has been strongly questioned.[53,54] In our current study, all flux rates were measured at concentrations that far exceed environmental concentrations and it is likely that carrier mediated transport processes were saturated. Here, we are measuring both the passive and facilitated uptakes, with passive uptake being dominant and entry likely via electrostatic interactions with the phospholipid membrane of the fish gill.[24] But several organic, anion, cation, or zwitterion transporters are present at the gill, e.g., *slco1d1*,[55] OATP,[56] and *slc15a2*,[57] and their ability to facilitate drug uptake from the water requires further understanding. An alternative explanation for the uptake of charged molecules is transportation as ion-pairs:[58] a property that has been utilized to assist in developing drug penetration for a number of epithelia, such as the ocular epithelium[59] and the skin,[60] but has not been considered for fish gill epithelia. Natural water contains numerous potential counterions, and the fish excretes ions and other charged molecules from the gill that could form ion-pairs with charged drugs.

■ ENVIRONMENTAL IMPLICATIONS

The current study shows that the FIGCS can be used to assess drug uptake by fish gills from the water in accordance with previous studies.[23] It also shows that ionizable drugs are able to cross the gill epithelium, but further work is required to ascertain the significance of the gill microclimate at the apical membrane with respect to ion pairing, electrostatic interactions (between the ionized pharmaceutical and the membrane phospholipids), and transport proteins on ionizable compound transport. A PLS regression model based on the physicochemical properties of the drug was used to predict uptake rate (the model accounted for 78% of the explained variance) where log *S*, pK_a, log *D*, and MW were significant drivers. To fully validate the model, a much larger number of compounds would be needed; however, this approach shows that modeling can be used to understand the uptake of pharmaceuticals by an in vitro epithelial system that could replace whole animals in bioaccumulation studies. There is a need for a robust and

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validated model for gill uptake that could then be used as a prescreen to prioritize compounds for experimental testing in a tiered risk assessment[8] where compounds that do not cross the gill epithelia may not need further costly and time-consuming animal testing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b04394.

> Cytotoxicity of the pharmaceuticals in primary gill cells (MTT assay), in vivo BCF data from literature, QSAR predictions of K_1, LC_{50}, and BCF, HPLC method performance assessment, linear regression analysis of uptake rates in relation to the chemical descriptors modeling the supporting information including scores, loadings, modified weights, and cross-validation for PCA and PLS (PDF)

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Notes

The authors declare the following competing financial interest(s): Dr Stewart F Owen is an employee of AstraZeneca, which is a biopharmaceutical company specializing in the discovery, development, manufacturing and marketing of prescription medicines, including some products reported here.

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